Microwave treatment of biological samples for methylmercury determination by high performance liquid chromatography—cold vapour atomic fluorescence spectrometry

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A simple and rapid microwave-assisted alkaline digestion procedure was developed in combination with high performance liquid chromatography—ultraviolet post-column oxidation—cold vapour atomic fluorescence spectrometric detection for methylmercury determination in biological tissues. Since the stability of methylmercury in methanolic potassium hydroxide solution under microwave irradiation was verified, the microwave-assisted extraction procedure was optimized in terms of quantitative recovery of methylmercury and minimum time required. The alkaline extracts were subjected to clean-up steps with dichloromethane and hydrochloric acid in order to reduce matrix interferences in methylmercury determination. The effects of matrix interferences were checked by comparison of the slopes corresponding to calibration and standard addition curves. The accuracy of the method was evaluated by the analysis of two biological certified reference materials, NRC TORT-2 and BCR 463. The results obtained by the proposed method were in good agreement with the certified values of methylmercury concentration in both materials. The detection limit was 10 μg kg⁻¹ and the relative standard deviation was <8% for methylmercury concentrations ranging from 0.15 to 3.0 mg kg⁻¹.

Introduction
The presence of mercury species in aquatic food chains was recognized long ago as a major environmental pollution issue and health hazard for humans. Inorganic mercury may be converted into the more toxic methyl mercury by aquatic organisms and be bioaccumulated in the food chain. As a consequence, fish usually contain high levels of methylmercury with increasing concentration in the tissue with the trophic level and age of the fish. Methylmercury usually represents more than 85% of the total mercury present in fish. Mercury speciation in fish samples is required because of the poisoning risk associated with the consumption of fish contaminated with methylmercury. The most widely used analytical methodology for mercury speciation determination in biological samples usually involves several steps, including extraction of mercury compounds and chromatographic separation of the different species followed by element specific detection. The extraction of methylmercury from a complex matrix is a limiting step because of incomplete extraction, species transformation, sample contamination or analyte losses that can be produced during sample treatment. Hence, the efficiency, precision and accuracy of the overall method are mostly associated with the sample preparation procedure. The Westoii method, based on successive extractions and back-extractions with organic solvents and an aqueous complexing agent, is the most commonly used procedure for the extraction of methylmercury from biological samples. Other isolation methods are also applied to the extraction of organomercury compounds from biological tissues, such as acid leaching or alkaline digestion using conventional heating sources or using steam distillation or ultrasonic energy or supercritical fluid extraction to decrease the extraction time. The main disadvantages of the above procedures are the low recoveries reported for methylmercury, the number of extraction steps involved, the time required and the amount of solvent consumed. Microwave energy has recently been applied in the acceleration of methods for methylmercury extraction from biological samples. Tseng et al. developed a rapid and simple microwave-assisted alkaline digestion method with 25% v/v trimethylammonium hydroxide for speciation determination of inorganic mercury and methylmercury in biological tissues by gas chromatography—electrothermal atomic absorption spectrometry (GC-ETAAS). Quantitative recoveries were obtained for an applied power of 40–60 W and a heating time of 2–4 min. The same extractant (25% v/v trimethylammonium hydroxide) was used by other workers for the acceleration of inorganic mercury and methylmercury extraction from biological tissues in a microwave field at 45 W for 2.5 min and at 120 W for 20 min and at 20 W for 20 min. Both mercury species were subsequently determined by gas chromatography—microwave induced plasma atomic emission spectrometry (GC-MIP AES), high performance liquid chromatography—atmospheric pressure

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Analyt., 2001, 126, 1583–1587 1583
ionization-mass spectrometry (HPLC-APCI-MS) and GC-MIP-AES, respectively. Vázquez et al.31 described the use of toluene in hydrochloric acid medium as an extractant for the complete extraction of methylmercury from fish tissues. Quantitative recoveries were obtained when the slurry were subjected to microwave irradiation for 10 min. Methylmercury determination was carried out by gas chromatography with electron capture detection (GC-ECD).

The most common method for mercury speciation in biological tissues is based on a derivatization reaction to form volatile mercury species, which are then separated by GC and detected with an element specific detection method such as MIP-AES,32-35 AAS,17,22 cold vapour atomic fluorescence spectrometry (CV-AFS),23-25 or inductively coupled plasma mass spectrometry (ICP-MS).12,13 Nevertheless, methods which rely on HPLC have also been used for mercury speciation in biological samples coupled to ICP-MS,12,13,26,28,29 flame photometry,31 cold vapour atomic absorption spectrometry (CV-AAS),13,12,13,33 or CV-AFS.30,32

The purpose of this work was to develop a simple, rapid and reliable microwave-assisted alkaline digestion method which permits the complete extraction of mercury compounds from fish tissues. The microwave-assisted extraction method was optimized in order to achieve quantitative recovery for methylmercury in a minimum time using 25% v/v methanolic potassium hydroxide as extractant. Organomercury species were subsequently extracted with dichloromethane and back-extracted into ultra-pure water in order to clean up the alkaline extract. Methylmercury was determined in the aqueous extract by high performance liquid chromatography—ultraviolet post-column oxidation—cold vapour atomic fluorescence spectrometry (HPLC-UV-PCO-CV-AFS). The combination of an alkaline digestion procedure with an HPLC-CV-AFS system for mercury determination in biological tissues has not previ-
ously been reported in the literature. The behaviour of methylmercury in alkaline solution under a microwave field was also investigated in terms of how to prepare the analyte identity and avoid analyte losses. The overall methodology was validated by the analysis of two certified reference materials of fish tissues containing different amounts of methylmercury.

Experimental
Institutionation

The chromatographic system used in this work was as described by Ramalho et al.31 Briefly, the system included an HPLC pump module (Kratos, Berlin, Germany), a six-port injection valve (Perkin-Elmer, Norwalk, CA, USA) equipped with a 200 μl PEEK sample loop and a reverse-phase analytical column packed with Nucleosil ODS (RPCe, 25 cm × 4.6 mm id, 5 μm particle size). All separations were performed at laboratory temperature under isocratic conditions. The post-column oxida-
tion system consisted of a UV-avaliation lamp (8 W, 254 nm) (Camag, Muttenz, Germany) surrounded by a 3-J coil. A reduction coil (2 m), two flow meters and a quartz gas—liquid separator cell (PS Analytical, Kent, UK) were used for mercury cold vapour generation. The solutions were introduced into the system by four-channel peristaltic pumps (Ismatec, Zürich, Switzerland) through Tygon tubes (K 5601). The mixing joints and both reaction coils were made of 0.50 mm id Teflon. The drying of the mercury vapour generated was carried out in a stainlesssteel guard trap (0°C) connected to a calcium chloride trap (7 cm × 1 cm id). A PS Analytical Model 10.023 Merlin atomic fluorescence spectrometer was used as a mercury detector. A CEM MDS-81D microwave oven (600 W maximum output) with glass tapers of 22 ml and Gilson (Villiers le Bel, France) thakers were used for sample preparation.

Reagents and reference materials

All standards and reagents were prepared in ultra-pure water produced using Milli-Q Model 185 system. The chemicals were of analytical-reagent grade and mercury free and were used without further purification. The mobile phase was a mixture of methanol (liquid chromatography grade, Merck, Darmstadt, Germany) (5% v/v) and ultra-pure water, containing 0.01% v/v 5-azauracil (Merck) buffered at pH 5 with 0.06% v/v acetic acid (Merck) and 0.15% m/v ammonium acetate (Merck). The mobile phase was filtered through 0.2 μm membranes (NL, 16, Schleicher & Schill, Dassel, Germany) and <1 μl in an ultrasonic bath for 30 min prior to use. The reducing agent, 3% m/v tin(II) chloride in 15% v/v hydrochloric acid, was prepared daily by the dissolution of the appropriate amount of mercury-free tin(II) chloride (Merck) in mercury-free hydrochloric acid (Merck) on a hot-plate. The solution was diluted to volume with ultra-pure water, filtered through 0.45 μm membranes (Millipore, Bedford, MA, USA) and purified from mercury by boiling with nitrogen for 2 h. Stock standard solutions of mercury nitrate (1000 mg l−1, Spectrosc, BDH, Poole, Dorset, UK) and methylmercury chloride (1000 mg l−1 in mercury, Alpha Products, Karlsruhe, Germany) were used weekly to prepare a working standard solution of 10 mg l−1 (as Hg) of each individual species in ultra-
pure water. Lower concentration working standard solutions were prepared daily in ultra-pure water. They were stored under refrigeration at 4°C.

A solution of 25% v/v methanolic potassium hydroxide was prepared daily by the dissolution of potassium hydroxide (Merck) in methanol (liquid chromatography grade, Merck). Dichloromethane was obtained from Merck (liquid chromatog-
raphy grade). Two biological reference materials with different certified contents of methylmercury were used to validate the proposed method. BCR 463 (Tuna Fish) was obtained from the Community Bureau of Reference (BCR) and TORT-2 (Lobster Hepatopancreas) was obtained from the National Research Council of Canada (NRC). The biological extracts were filtered through 0.2 μm membranes (PVDF PP, LIDA, Krefussa, USA) prior to injection.

Figures of merit

The analytical performance of the HPLC-UV-PCO-CV-AFS technique was in accordance with methylmercury and inorganic mercury standards. The retention times were 19 and 24 min for methyl/mercury and inorganic mercury, respectively. The detect-
ion limits of methylmercury (M) and inorganic mercury (I) were calculated from calibration curves constructed in the range 100–800 ng l−1 and based on the amount necessary to yield 4 signal equal or 3.3 times the standard deviation of the blank. The relative standard deviation (n = 4) for a 300 ng l−1 methylmercury standard was <1%.

Extraction procedure

Samples of 0.15 g were weighed into glass tubes, 6 ml of 25% m/v methanolic potassium hydroxide were added and the tubes were capped. The slurry was homogenized by magnetic agitation and submitted to microwave irradiation for 30–180 s at 48–132 W. The digestate was allowed to cool in laboratory temperature. The alkaline extract was mechanically shaken in a glass separating funnel for 10 min with 5 ml of chloroform and 4.5 ml of concentrated hydrochloric acid. With this procedure organomercury compounds were extracted into the organic phase, whereas inorganic mercury remained in the aqueous phase as chloro complexes. An aliquot of dichloro-
methane was transferred into another tube and the slurry was again treated with 6 ml of dichloromethane for 10 min. Finally, the dichloromethane layers were combined and organomercurials were solvent exchanged into 35 ml of ultra-pure water by evaporation of the organic solvent with a current of nitrogen. This final solution was injected into the HPLC-UV-PDO-CV-APFS system. Blanks and methylene mercuacy standards were subjected to the same procedure in order to check for possible contamination and any type of losses or interconversion of species, respectively.

Results and discussion

The use of concentrated acids for methylene mercuacy extraction from biological samples can cause evaporation losses, methylene mercuacy degradation to inorganic mercury or insufficient digestion of the sample leading to low recoveries of methylene mercuacy. However, several procedures based on alkaline digestion were found to be effective for methylene mercuacy extraction from biological tissues. Since several hours (3–24 h) have been reported22,41 as the time required for dissolving the sample in alkaline solutions, other workers13,43 considered the use of sonication to reduce the time required for alkaline digestion (2–3 h). Recently, microwave-assisted alkaline digestion methods permitted the quantitative recovery of methylene mercuacy from biological tissues in a few minutes.17,18,20 Most of the alkaline digestion procedures for dissolution of biological tissues were used in combination with GC or GC coupled to different element specific detectors. In this work, methylene mercuacy determination in purified alkaline extracts of biological tissues was carried out by HPLC-UV-PDO-CV-APFS, since this coupled system has not been reported previously, to our knowledge.

Methylene mercuacy stability in a microwave field

The behaviour of methylene mercuacy in alkaline solution under microwave irradiation was investigated. For this purpose, 6 ml of 25% m/v methanolic potassium hydroxide was spiked with 7.5 ng of methylene mercuacy and divided into two portions. The first portion was microwave irradiated at an applied power of 48–132 W for 5 min and the second portion was not exposed to microwave irradiation. Although similar experiments have already been carried out,21 the power and time ranges were different to those in this work. All experiments were performed in two replications and each replication was measured twice. The methylene mercuacy recovery, calculated for each microwave power as the ratio of the methylene mercuacy concentrations determined with and without microwave treatment, was >95% at all applied powers. Additionally, no inorganic mercury signal was encountered in either of the chromatograms obtained. The results showed that neither evaporation losses nor methylene mercuacy decomposition to inorganic mercury were produced when the samples were heated in a domestic microwave oven. In consequence, 25% m/v methanolic potassium hydroxide can be considered as an appropriate medium for the microwave-assisted extraction of methylene mercuacy from fish tissues.

Optimization of microwave-assisted extraction procedure

In this work a series of experiments were carried out to optimize the microwave-assisted alkaline digestion procedure in terms of quantitative methylene mercuacy recovery and minimum exposure time, through the evaluation of the effect of parameters such as microwave power and exposure time. For this purpose, 0.15 g of a biological certified reference material, NBC TORT-2, was suspended in 6 ml of 25% m/v methanolic potassium hydroxide and submitted to microwave irradiation at an applied power ranging from 48 to 132 W for exposure times of between 30 and 180 s. All experiments were performed in two replicates and each replicate was measured twice. A six-point standard additions method was always used in the determination step in order to avoid possible matrix interferences. The effects of both parameters (exposure time and microwave power) on methylene mercuacy recovery (calculated as the ratio of determined and certified concentrations of methylene mercuacy) are shown in Fig. 1, where it can be observed that the methylene mercuacy recovery increased with increase in applied power for short exposure times. For exposure times longer than 80 s, the methylene mercuacy recovery decreased, probably owing to losses by evaporation. In consequence, the optimum conditions for the proposed procedure were an applied power of 80–90 W and an exposure time of 1 min. In order to ensure better precision and reproducibility between replicates, the use of sample amounts >0.15 g is possible. However, in such a case, two factors must be considered: (i) the relation between sample amount and volume of methanolic potassium hydroxide must be kept constant and (ii) the alkaline extract must be diluted in the same relation to avoid the occurrence of violent reactions between potassium hydroxide and hydrochloric acid, excessive formation of potassium chloride (which makes the phase separation difficult) and high consumption of dichloromethane and hydrochloric acid during the clean-up of the alkaline extract.

Matrix effects in biological tissue extracts

In order to check the effects of interferences on methylene mercuacy determination in the purified alkaline extracts of fish tissue by HPLC-UV-PDO-CV-APFS, the slopes of the calibration curves obtained by adding different amounts of an aqueous standard of methylene mercuacy to either ultra-pure water or fish tissue extracts were statistically compared. A historical series of analytical values was plotted (Fig. 2), which included aqueous standards,
Table 1 Slope and intercept values corresponding to the calibration curves for aqueous standards and biological tissue extracts spiked with methymercury

<table>
<thead>
<tr>
<th>Sample</th>
<th>Slope (ng/ml) ± SE</th>
<th>Intercept (ng/ml) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous standard (n = 74)</td>
<td>19.143 ± 2.036</td>
<td>0.388 ± 0.624</td>
</tr>
<tr>
<td>Aqueous standard (n = 75)</td>
<td>20.345 ± 2.036</td>
<td>0.388 ± 0.624</td>
</tr>
<tr>
<td>BCR 463 (n = 75)</td>
<td>14.371 ± 1.373</td>
<td>3.077 ± 0.785</td>
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</tbody>
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Conclusions
The proposed microwave-assisted extraction procedure shortened the time for alkaline digestion of biological tissues with 25% methanolic potassium hydroxide from 3 to 1 min in comparison with other work published previously,3,32 and also achieved the quantitative recovery of methymercury. Furthermore, this sample treatment procedure offers the possibility of conducting several simultaneous extractions. The alkaline extracts were analysed by HPLC-UV-PCO-CV-AFS and the overall method was validated through the analysis of two biological certified reference materials. The results obtained confirm the efficiency, reproducibility and accuracy of the proposed method. The detection limit obtained, 0.1 µg kg⁻¹, was similar to those in other work reported with the combination of alkali digestion with GC coupled to AAS,7,12 AFS30 or AES.13

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